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13. ABSTRACT (Maximum 200 Words)

Cyclin E is a positive regulator, which controls the transition of the G1 to S phase of the cell cycle. When associated with CDK2, it is responsible for cells passing through the restriction point, which is the barrier between G1 and S. This commits the cell to complete one round of cell division. Previous findings by this laboratory have found that overexpression of cyclin E and the presence of lower molecular weight isoforms (LMW) are found more often in breast tumors and cancer cell lines when compared to normal tissues and cells. Also, tumor cells, but not normal cells have the mechanisms to proteolytically cleave the full length cyclin E into these LMW forms. An altered cyclin E may contribute to the deregulation of the G1 to S checkpoint and lead to tumorigenesis. Our laboratory has also identified through mutational and biochemical analysis, the region of cyclin E that is proteolyticaly cleaved to generate the LMW forms. Critical phosphorylation sites of cyclin E are responsible for the appearance of the LMW forms of cyclin E. To investigate the possible role of phosphorylation in the processing of cyclin E into these lower forms, two approaches have been employed. First, incubation of breast cancer cell line extracts expressing the LMW forms with phosphatases was examined via western blot analysis. Visualization of cyclin E showed downward shifts in both the full length and lower forms in the presence of active dephosphorylation. Second, full length and truncated cyclin E cDNAs were mutated at critical phosphorylation residues via site-directed mutagenesis. Transfection of these mutants into tumor cells capable of LMW form processing followed by western analysis indicates that at least one of the mutations results in the loss of 2 lower forms of cyclin E. However, assays for kinase activity in the transfected tumor cells demonstrate no change activity of the mutants deficient in these phosphorylation sites.

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Table of Contents

Introduction:

One of the first steps in the multi-step process of tumorigenesis is the deregulation of the cell cycle, which can cause the cells to replicate uncontrollably. Many cancers have been associated with the abnormal expression of proteins involved in the regulation of the cell cycle. Alterations of cyclin E, a positive regulator of the G1 to S phase transition, have been found in several types of cancer, including breast carcinomas. Furthermore, in breast cancer patients, there is a correlation with overexpression of cyclin E and the lower molecular weight (LMW) forms and poor patient prognosis. Also, tumor cells, but not normal cells have the mechanisms to proteolytically cleave the full length cyclin E into these LMW forms. An altered cyclin E may contribute to the deregulation of the G1 to S checkpoint and lead to tumorigenesis. Our laboratory has also identified through mutational and biochemical analysis, the region of cyclin E that is proteolyticaly cleaved to generate the LMW forms. Critical phosphorylation sites of cyclin E are responsible for the appearance of the LMW forms of cyclin E. Characterizing the processing of the LMW forms and exploring the processing mechanism will address their role if any, in breast cancer tumorigenisis.

Body:

To investigate the possible role of phosphorylation in the processing of cyclin E into these lower forms, we first incubated breast cancer cell line extracts expressing the LMW forms with phosphatases and they were examined via western blot analysis. Figure 1 is a representative example of cycE LMW forms in breast tumor cell line MDA-MB 436.

EL1: full length cyclin E

EL2/3: Doublet formed; proteolytic processing in residues 40-45 (45/44kD)

EL4: Formed via alternate start site Met46 (365aa, 40kD)

EL5/6: Doublet formed via proteolytic processing at aa 70 (346aa, 35/33 kD)

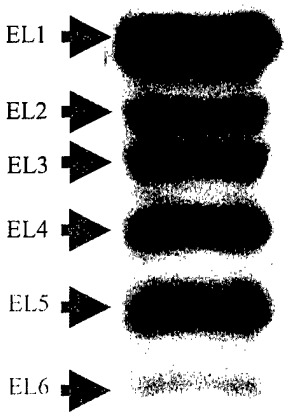
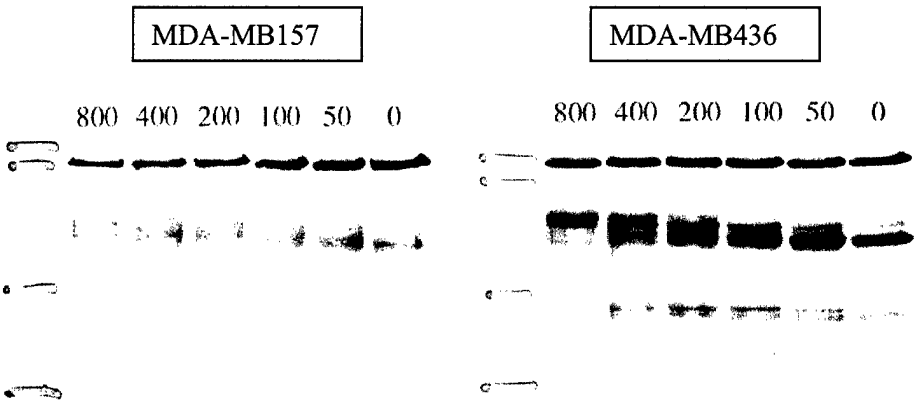


Figure 1. *Cyclin E LMW forms in the breast cancer cell line MDA-MB231.*

Two breast tumor cell line extracts (MDA-MB 436 and 157) were treated with λ-phosphatase (λPPase) at several enzyme concentrations and then analyzed via western analysis. Detection of cyclin E shows changes in mobility of the full length (EL-1) and lower forms in both cell lines (fig. 2). Enzyme activity was confirmed by detection of E2F-4, a hyperphosphoryated protein found in these extracts.

Figure 2. *λ-Phosphatase treatment of breast tumor cells.*



Cyclin E cDNAs constructed in our lab [1] give rise to different length proteins when transfected into cells capable of the processing seen in tumors. Transfection of EL produces all six forms shown in figure 1. Trunk1, which has a start fused at residue 40, displays all the forms except E1. Trunk2 only gives rise to E5 and E6. To directly assess the role of phosphorylation in the appearance of LMW forms, these constructs were mutated at critical (ubiquitination/degradation) phosphorylation residues, T395 and T77 [2]. Single and double mutants were generated via site-directed mutagenesis in all three cDNAs. Transfection of normal and mutated constructs into processing-competent cell line 293T shows that the mutation of T395->A causes loss of forms E2 and E5 (fig. 3). T77->A showed no change. Similar results were obtained in MDA-MB 436 cells.

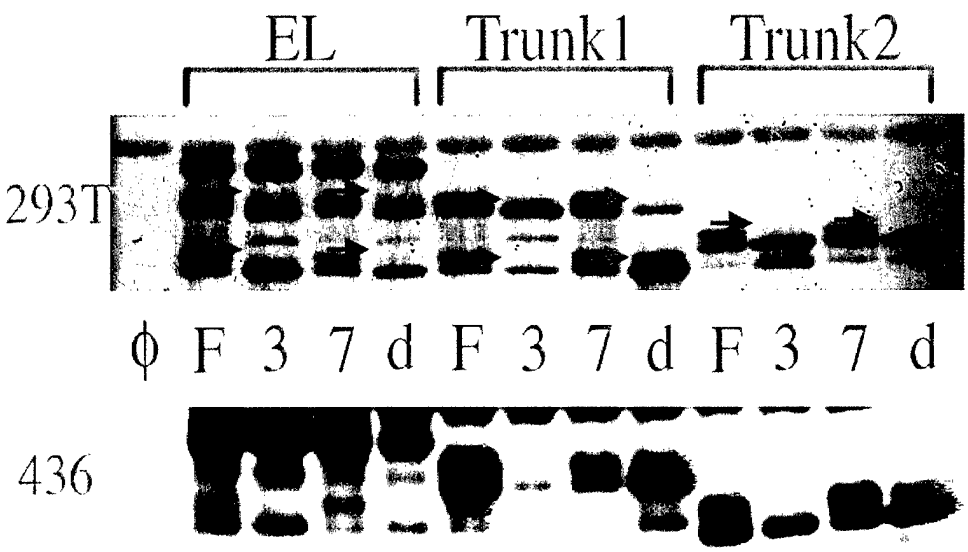


Figure 3. *Transfection of mutants into 293T cells and MDA-MB436.* IP western analysis of 293T (top) and MDA-MB 436 (bottom) cell lines transfected with wild type and mutant EL, Trunk1 & Trunk2 cycE constructs. Anti-FLAG probe detects exongenous cycE.

To analyze functional differences in the mutated cyclin E constructs, histone H1 kinase assays were performed on the 293T extracts described above. Results for all three constructs in 293T showed no significant change in activity in the mutant forms (fig. 4). Similar results were obtained in MDA-MB 436 cells transfected with the EL constructs.

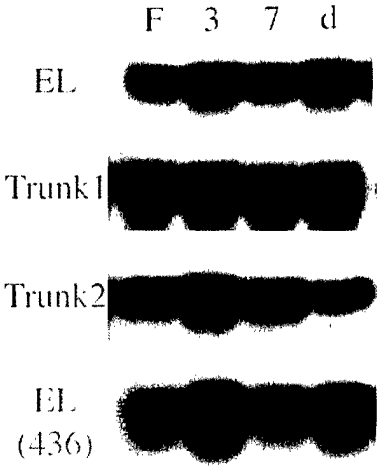


Figure 4. *Histone H1 kinase assay of mutant cycE in 293T cells and MDA-MB436.* IP kinase assay on 293T (top 3 panels) and MDA-MB 436 (bottom panel) cell extracts using histone H1 as substrate.

To determine whether cyclin E is truly autophosphorylated by the cycE/cdk2 complex, unmodified/synthesized cyclin E was used as a substrate for kinase activity of purified cycE/cdk2. Unmodified cyclin E was generated using the LMW constructs in an *in vitro* transcription/translation system (Promega). Kinase activity of purified cyclin E/cdk2 produced in Sf9 insect cells [3] using the *in vitro* cyclin E products was analyzed. The cyclin E/cdk2 was able to phosphorylate El, Trunk1 and Trunk2. To determine if

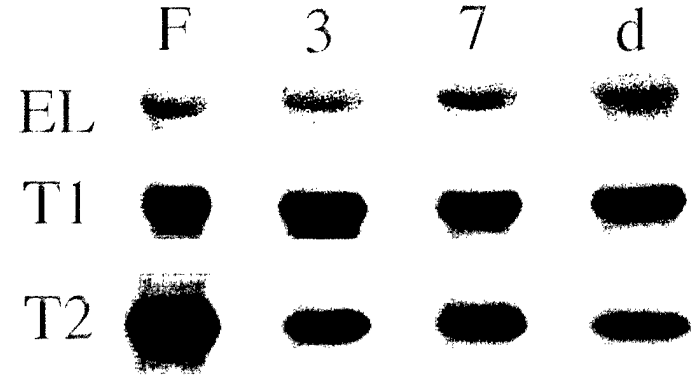


Figure 5. *Kinase reaction of CycE/cdk2 on in vitro TNT Cyclin E and mutants.* Kinase reaction of IP cycE/cdk2 using *in vitro* transcribed/translated cycE wild type and mutants as substrate.

the mutations described above would alter this activity, *in vitro* products were generated for all mutants and kinase activity was analyzed (fig 5). The

mutations at T395 and T77 had no effect on the degree of phosphorylation by cyclin E/cdk2. These results show that while cyclin E is a substrate for the cycE/cdk2 complex, the sites mutated in this assay do not prevent autophosphorylation.

Key Research Accomplishments:

• Showed phosphatase treatment causes a shift in the wild type and LMW forms of cyclin E

• Demonstrated that mutation of T395->A caused loss of the E2 and E5 forms of cyclin E; however, the mutation did not alter the kinase function of cyclinE/cdk2 in tumor cell lines

• Established cyclin E is directly phosphorylated by cyclin E/cdk2 *in vitro*; however degree of phosphorlyation by cyclin E/cdk2 is not affected by cyclin E mutations T395->A and T77->A

Reportable Outcomes:

Manuscripts in progress

Conclusions:

The lower molecular weight (LWM) isoforms of cyclin are only expressed in tumor cells and such overexpression of these forms is indicative of the stage of the disease. Cyclin E could be used as a strong prognostic indicator of correlation with overexpression of cyclin E and the LMW forms and poor patient prognosis. Furthermore, there is evidence that overexpression of the T2-form of cyclin E results in a decrease in the levels of the full length, wild type form of cyclin E, possibly indicating a role for the LMW forms in further proteolytic cleavage or degradation of the full length cyclin E. Phosphatase treatment of cycE causes a shift in full-length and LMW forms. Mutation of T395 results in loss of doublet LMW forms (E2 and E5), contrastly, mutation of T77 results in no mobility change. The loss of either or both phosphorylation sites has no effect upon kinase activity/substrate binding. While we have achieved a great deal of work in this research period, we would like the opportunity to continue our research to evaluate if binding and associated kinase activity of cycE phosphorylation-null constructs differs between normal and tumor cell lines and to determine if structural mutations of amino terminal residues alters predicted folding of cycE.

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